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(54) Title: SURFACE MEMBRANE PROTEINS AND THEIR EFFECT ON IMMUNE RESPONSE		
(57) Abstract		
p74 is a protein found in T-cells and other cells, which when bound with specific agents results in inhibition of cytolytic activity and differentiation of CTLs. p74 can be isolated from T-cells and other cells using palindromic HLA-B2702.84-75-84 peptide by affinity binding of a cell lysate.		

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SURFACE MEMBRANE PROTEINS AND THEIR EFFECT ON IMMUNE RESPONSE

INTRODUCTION

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Technical Field

The field of this invention is modification of immune response.

Background

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The immune system is the subject of ever increasing scientific scrutiny. Despite the enormous interest in the immune system and the continuously expanding number of investigators, both academic and industrial, a complete understanding of the system continues to remain elusive. One of the major breakthroughs was identification of the interaction between the T-cell receptor and a major histocompatibility complex antigen.

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The identification that both class I and class II major histocompatibility complex antigens have a cleft which binds a small peptide provided a significant key to understanding T-cell specificity and T-cell restriction.

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The understanding that the MHC served macrophages and B-cells in their role as antigen presenting cells, where the peptide served to define which T-cells could bind to the MHC-peptide complex still did not explain many other aspects of the T-cell response. It appeared that the binding of the T-cell receptor to the antigen presenting cell was not enough by itself to result in T-cell activation. The process of anergy or tolerization could not be explained by an activation process solely involving the T-cell receptor and the MHC antigen. In addition, there was the distinction between helper cells (CD4+) and suppressor/cytotoxic cells (CD8+). Some mechanism was necessary to associate the helper cells with class II MHC and the suppressor/cytotoxic cells with class I MHC. It was subsequently found that CD4 and CD8 participated in the MHC TCR complex by binding a loop of the MHC, enhancing the stability of the complex.

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In addition, other interactions were uncovered, apparently not directly associated with the T-cell receptor/MHC complex, where CD28 and B7 were found to bind. Thus, there appear to be a number of different interactions involved with the association of the antigen presenting cell and the T-cell, which could result in tolerization or activation of the T-cell.

Because of the crucial role that the T-cell plays at the center of a major component of the immune system, it remains of great importance to be able to understand how T-cells are selected, activated or tolerized. By understanding the role that various participants play in T-cell activation, there will be opportunities to regulate the immune system, either enhancing the immune response, where one is dealing with vaccines, pathogens, neoplasia, or the like, or diminishing the immune response, where one is dealing with autoimmunity or organ transplantation.

A number of immunosuppressants have been shown to have specific cellular proteins as targets. Cyclosporin A binds to cyclophilin, FK506 to FK binding protein, and deoxyspergualin to both Hsc70 and Hsp70. Hsp 70 has been shown to be a member of a multigene family, having at least one constitutively expressed cognate, Hsc70. The effect of DSG seems to be specific for antigen presenting cells; interfering with antigen presentation and/or processing. The action of immunosuppressants is mediated by their cellular binding proteins, and there is, therefore, interest in identifying targets and other agents binding to the targets which will provide modulation of the immune response.

Relevant Literature

Wan *et al.* (1986) J. Immunol 137:3671-4 describes the Bw4/Bw6 epitopes. Clayberger, *et al.* (1987) Nature 330:763-765 demonstrates that HLA-A2 peptides can regulate cytotoxicity by human allogeneic T lymphocytes. WO93/17699 describes the activity of peptides from the HLA-A and -B $\alpha 1$ and $\alpha 2$ helices in modulating CTL activity. HLA-B2702.60-84 peptide is described therein.

The molecular characterization of the heat shock protein Hsp 70 is described in Immunogenetics (1990) 32:242-251, and its constitutively expressed homologue in Mol. Cell. Bio. (1988) 8:2925-2932.

Characterization of the immunosuppressive effect of deoxyspergualin (DSG) may be found in Hoeger, *et al.* (1994) J. Immunol. 153:3908. The binding of DSG to Hsp70 and Hsc70 is described in Nadler *et al.* (1992) Science 258:484 and Nadeau *et al.* (1994) Biochemistry 33:2561.

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SUMMARY OF THE INVENTION

Methods are provided for preparing and isolating T-cell proteins which participate in T-cell activation upon antigen presenting cell - T-cell interaction. The proteins are characterized by being associated with T cell activation and binding to the HLA-B2702 α 1 helix peptide, and are immunologically cross-reactive with the heat shock protein cognate, Hsc70. The proteins, fragments thereof and nucleic acids encoding the proteins and oligonucleotides are provided, as well as antibodies which bind thereto. By employing agents which bind to the proteins or inhibit binding of other agents to the proteins, T-cell activation may be modulated.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Methods and compositions are provided for isolating proteins expressed by T cells as surface membrane proteins associated with T-cell activation in mammalian T-cells, which proteins bind to the HLA-B2702 α 1 helix peptide, nucleic acid encoding such proteins or fragments thereof, and agents for modulating the signal transduction associated with the proteins in the modulation of activation of T-cells.

The protein of interest is referred to as p74. It is a surface membrane protein which is expressed in T cells and a limited number of other cells. It can be readily isolated by extracting a lysate of T cells with the peptide of the α 1-helix of HLA-B2702, namely the RENLRIALRY sequence, peptide extensions thereof, or dimers thereof, particularly palindromic dimers, having the amino acid sequence YRLAIRLNERRENLRIALRY. The affinity of p74 for the HLA-B2702 palindrome is at least about 10^{-4} M. The extraction may occur with the peptide on a surface, such as particles, microtiter well walls, etc. or in solution, where the peptide may be labeled with a label which allows for separation of complexes with the peptide. For example, one may label the amino terminus of the peptide with a ligand, or biotin and precipitate or sequester complexes with antibody or avidin, respectively. The protein may be freed from the peptide and characterized as appropriate.

p74 is a protein found in a limited number of cells, particularly activated CTLs, the T cell tumor PEER, and the EBV transformed cell line JY, in effect, lymphoid T and B cells. The p74 protein can be obtained by lysis with an amphoteric detergent, such as CHAPS. The lysate may then be combined with a peptide or protein having an appropriate Bw4 epitope, for example, HLA-B 2702.84-75-84 palindromic peptide. By preparing an affinity column, having the peptide covalently bound to appropriate particles, the lysate may be passed through the column, followed by elution with lysis buffer or other convenient buffer. Effective elution can be achieved using relatively

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high pH, conveniently in the range of about 11-12. See, for example, (Ey (1978) *Immunochemistry* 15:429-436). Alternatively, a different detergent may be used as described by Mescher *et al.* (1983) *Meth. Enzymol.* 92:86-109. Other separation techniques employing the peptides bound to a support may also be employed, such as panning, magnetic beads, or the like. Usually, after each contacting of the peptide with the p74 sample, the supernatant will be removed and the solid support washed to remove non-specifically bound proteins and other contaminants. Washes may be any convenient buffer solution, varying in salt content. The washing should not adversely affect the complex between the peptide and p74. In this way successive enrichments may be employed to provide for a protein composition having at least about 50 weight % of total protein as p74 protein, preferably at least about 75 weight %, more preferably at least about 90 weight %, up to 100 % pure.

As an alternative purification scheme, the immunological cross-reactivity of p74 with the heat shock protein cognate, Hsc70, may be exploited. Antibodies directed against Hsc70 react with p74. Various affinity purification methods are known in the art. Antibodies may be bound to beads or another insoluble support, and combined with the cellular lysate. The bound protein is washed free of the unbound lysate. The bound protein is eluted from the antibody by a number of methods, *e.g.* heat, salt gradients, mild detergents, *etc.*

Instead of affinity chromatography, gel electrophoresis of the lysate may be performed, where the Bw4 epitope containing peptide, suitably labeled, may be used to identify the presence of p74. The Bw4 peptide may be labeled with fluorescer, radioisotope, or the like, non-specifically bound peptide washed away, and the appropriate band developed.

Instead of binding on a solid or in a gel, binding can be accomplished in solution, using a suitably labeled soluble form of the peptide, *e.g.* N-terminal biotinylation, *etc.* By incubating an appropriate cell with the peptide or dimeric form thereof, particularly palindromic form, at a mildly elevated temperature, usually about 25 - 40°C. for sufficient time for complexes to form, one obtains specific binding of the peptide to cell surface proteins. The complexed cells may then be lysed as described above. After lysis, the complexes may be isolated by means of the label, *e.g.* biotin, isolated by means of avidin bound to a solid surface. Alternatively, the label-complementary binding member complex may be preformed and used to extract specific protein binding to the peptide as described above. The samples may then be washed extensively, boiled in SDS and separated using SDS-PAGE gel electrophoresis.

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Prior to the affinity purification or gel electrophoresis, one may enrich for the desired p74 protein by using the peptide sequence of the B7.84-75-84 palindrome, YGRLNRLSERRESLRNLRGY, to remove any proteins which bind to that sequence, as distinct from the B2702.84-75-84 palindrome sequence to which the p74 binds. In this manner, p74 may be greatly enriched and obtained substantially free of other proteins.

The subject proteins may be obtained from any mammalian species, such as rodent, bovine, canine, primate, particularly human, or the like.

Using the proteins individually in a sample of at least about 80 weight % purity, based on protein, the sequence of the proteins may be readily determined in accordance with conventional ways. The protein purified as described above may be sequenced using conventional sequence equipment. The purified protein may be cleaved, using mild proteolytic degradation, cyanogen bromide, or other mode of cleavage, to provide fragments under about 20 amino acids. The fragments may then be fractionated *e.g.* reverse phase-HPLC or preparative SDS-PAGE and electroelution, and the fragments sequenced. The sequences of the fragments may then be used to deduce the sequence of the respective protein.

By employing conventional techniques, degenerate probes may be produced from the known amino acid sequence of the proteins. Using DNA probes of at least about 15 nucleotides, usually of at least about 20 nucleotides, one may screen a cDNA library of cells known to express the proteins, particularly T-cells. Those cDNAs which bind to the probes may then be isolated and sequenced. Where the sequence encodes the appropriate peptide sequence, if the isolated cDNA is not a complete cDNA, the isolated cDNA may be used as a probe to isolate a complete cDNA which encodes the entire protein. If desired, the cDNA gene may then be used to isolate the genomic gene in accordance with conventional techniques. (See, for example, *Molecular Cloning: A Laboratory Manual*, 2nd ed., J. Sambrook, E. F. Fritsch, T. Maniatis, CSHL, Cold Spring Harbor, NY, 1989).

The gene or fragments thereof, usually of at least about 25 nucleotides, preferably of at least about 30 nucleotides and not more than about 60 nucleotides, may be used for probing cells to determine whether a subject protein is expressed, when in the cell cycle the protein is expressed, and at what level of differentiation it may be expressed, and quantitate the amount of message RNA during activation or deactivation of T-cells.

The gene may be introduced into an expression vector for expression in a wide variety of hosts, both prokaryotic and eukaryotic. A large number of promoters are

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commercially available today which are included upstream (in the direction of transcription) of a polylinker for insertion of a gene to be under the transcriptional and translational control of a transcriptional and translational initiation regulatory region functional in an expression host. Besides the transcriptional and translational initiation regulatory region, the expression vector may include downstream from the polylinker, a transcriptional and translational termination regulatory region, functional in the expression host. Thus, the gene may be inserted into the vector after linearization of the vector with a restriction enzyme which cuts at a convenient site in the polylinker. In addition to the transcriptional and translational regulatory regions, the expression vector may also include a gene which allows for selection of hosts comprising the expression vector. For the most part, these genes will provide for antibiotic resistance, but may also serve to complement an auxotrophic host, or other mode of selection. The vector may also include an origin for episomal maintenance or the vector may allow for integration into the expression host. The vector may be introduced into the expression host by any convenient means, including electroporation, fusion, calcium precipitated DNA, transfection, *etc.*, the particular mode employed not being critical to this invention and depending upon the choice of expression host.

In addition to using the expression construct for production of the subject proteins, the expression construct can also be used in the elucidation of T-cell activation. These constructs may be used, for example, in complementation studies. For example, cells lacking one or both of the subject proteins, as well as other surface member proteins associated with T-cell activation, may be modified so as to express one or both of the subject proteins. The effect of p74 binding on various pathways in the cell may then be studied. These studies may also examine the effect of mutations on the activity of p74. In addition, one may choose to use the cytoplasmic portion of p74 and bind it to a different extracellular portion, so as to provide a fused protein, which will transduce the signal when a ligand or other binding molecule for the extracellular portion binds to the extracellular portion. For example, one may use various receptors with known ligands, such as the EGF receptor, IL2 receptor, insulin receptor, CD4, or the like, where binding of the protein, particularly crosslinking on the surface, will result in signal transduction. Thus, one may use a natural ligand or antibodies to the receptor, so as to provide for the signal transduction. A soluble form of p74 may be constructed by deletion of the transmembrane region. The soluble protein is used as a competitor for the native protein, thereby modulating the cellular immune response.

The expression constructs may also be used to produce fusion proteins, where p74 may be fused to a marker, such as β -gal, CAT, *lacZ*, and the like. The fusion

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proteins may serve as markers for translational expression, for production of antibodies, in assays, or the like. The fusion proteins may serve to aid in the purification of a source of p74, where p74 or truncated portion thereof may be joined to a sequence which allows for purification in the Qiagen process or other process.

- 5 p74 binds to HLA-B 2702.75-84, particularly as the palindromic dimer, and not to HLA-B7 75-84 or its palindromic dimer. It is associated with the process of T-cell activation. Binding to p74 can result in inhibition of cytotoxicity by CTL as well as CTL differentiation. By employing agents which bind to the p74 of CTL, the immune response can be significantly inhibited. Alternatively, by inhibiting binding agents, 10 either naturally occurring or synthetic to p74, CTL activity may be maintained, other aspects necessary for CTL activity being present. For inhibition of CTL activity, peptides having the Bw4 epitope may be employed in an amount sufficient to inhibit differentiation or cytotoxicity of target cells. Alternatively, antibodies may be employed which bind to p74, where the antibody may provide for activation or inhibition. 15 Depending upon the antibody, it may inhibit binding of an agent to p74 allowing for activation of the CTL, it may provide for activation of the CTL in conjunction with other interactions of surface member proteins or other signals being transduced into the cell, or it may independently inactivate the CTL through the binding of p74. Whole antibodies need not be used, binding fragments thereof, such as Fab, F(ab')₂, Fv, or 20 the like, or other binding entities, either naturally occurring or synthetic.

- p74 when bound to the B2702 peptide results in a large calcium influx in nontransformed T cells. The magnitude and kinetics are essentially the same as that induced by anti-CD3 antibodies, supporting the conclusion that the rise in intracellular calcium is involved in the development of anergy. This response appears to be specific 25 to the B2702.75-84 peptide.

- Soluble p74 may be prepared by truncating the respective gene and removing the transmembrane sequence and the intracellular sequence. The truncation can be achieved in accordance with conventional methods, using restriction enzymes, primer repair, exonucleases, or synthesis of the extracellular portion of the protein. The 30 soluble p74 may be used for a variety of purposes. The soluble p74 may be used in diagnostic assays to determine the presence and amount of p74 in a sample, may be used for screening compounds which bind to p74 in accordance with conventional binding assays, for prophylactic or therapeutic purposes (i.e. to inhibit binding of agents to the native protein, as well as in research to elucidate the mechanism of T cell 35 activation.

Soluble p74 may be used for identifying APCs which bind to p74 as well as agents which interfere with such binding. Using labeled p74, cells binding to p74 can be readily detected by magnetic separation, fluorescence, *etc.* Soluble p74 may also be used to modulate CTL response *in vivo* or *in vitro* in a mixture of T cells and antigen presenting cells, such as macrophages and B cells.

Antibodies to the subject proteins may be prepared in accordance with conventional methods. p74, native or soluble form, may be used as an immunogen, being injected intravascularly, intraperitoneally, intramuscularly, subcutaneously, or the like normally in conjunction with an adjuvant, into an appropriate mammal, *e.g.* mouse, rat, guinea pig, or the like. Usually, one or more booster injections will be employed to enhance the specificity of the antisera. The immunized host may then be sacrificed, the spleen isolated and splenocytes immortalized by any convenient means, *e.g.* fusion with a hybridoma. The hybridomas may then be grown using limiting dilution and screened for binding characteristics with p74, as appropriate. Hybridomas which show binding affinity for p74, may then be expanded and the supernatant used for isolation of monoclonal antibodies, or the hybridomas used for the preparation of ascites fluid as a source of monoclonal antibodies. The hybridomas may then be further subcloned and screened to identify antibodies which have a desired binding affinity. These antibodies may be then be further screened with T-cells having p74 under conditions where the physiological effect of the antibodies can be determined. For procedures for preparing monoclonal antibodies, see *Antibodies: A Laboratory Manual, Eds., Ed Harlow and David Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988.*

For use as prophylactic or therapeutic reagents in a host, the antibodies may be modified by replacing constant regions and framework regions of the antibody with sequences utilized by the host to be treated. Techniques for isolating the variable regions of the heavy and light chains and fusing them to variable regions native to the host, as well as substitution of framework regions native to the host, have been described in numerous publications. See, for example, EPA 173,494 and WO92/16562.

The subject therapeutic agents may be prepared as formulations in pharmaceutically acceptable media, for example saline, PBS, in glucose, generally at a pharmacologically effective dose, the concentrations of which will be determined empirically in accordance with conventional procedures for the particular purpose. The formulations may include bactericidal agents, stabilizers, buffers or the like. The amount administered to the host will vary depending on what is being administered, the

purpose of the administration, such as prophylaxis or therapy, whether inhibition or activation is desired, the state of the host, the manner of administration, and the like. In order to enhance the half life of the subject peptide agents, the peptides may be encapsulated, introduced into the lumen of liposomes, prepared as a colloid, modified
 5 by binding to stable peptides, such as the constant region of IgG, or polyalkyleneoxy groups, or other conventional techniques.

The role of p74 in the mixed lymphocyte reaction, B cell activation, and other cellular or compound interactions may be investigated. For example, in culture, the p74 or biologically active fragment thereof may be added to the culture medium in from
 10 about 1 to 500 μg per 1×10^5 cells, where inhibition of T cell activation is studied. In addition, p74 may be used for screening novel compounds for agonist or antagonist activity to CTL activation. Thus, one can combine the protein bound to a peptide or other agent which binds to the protein, where the peptide or other agent is labeled, so as to be detectable. By carrying out a competition between the complex involving p74 and
 15 the labeled agent with the compound being analyzed, and determining the rate of release of the labeled agent from p74, one can measure the affinity of the candidate compound for p74. Alternatively, one may use CTLs where the peptide or other agent competes for binding to the protein with the candidate compound and measure the effect of the candidate compound on CTL differentiation or cytolytic activity as compared to a
 20 standard, where the candidate compound is not present.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

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Effect of a Variety of HLA Peptides of Amino Acids 60-84 and HLA-B 2702/05.145-169 on Lysis

The following peptides were synthesized:

HLA-B2702.60-84	WDRETQICKAKAQTDRENLRALRY
30 HLA-B2705.60-84	WDRETQICKAKAQTDREDLRLLRY
HLA-Bw46.60-84	WDRETQKYKRQAQTDREVSLRNLRGY
HLA-Bw62.60-84	WDRETQISKNTQTYRESLRNLRGY
HLA-A2.1.60-84	WDGETRQVKAHSQTHRVDLGTLRGY
HLA-B2702/05.145-169	RKWEAARVAEQLRAYLEGECVEWLR
35 HLAB38.6084	WDRNTQICKTNTQTYRENLRALRY

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The effect of the above sequences on lysis of long-term CTL specific for HLA-A2, -B2705, -Bw46, -Bw62, and -Cw4 was determined, including CTL specific for HLA-B27 and the HLA-Cw4. None of the peptides inhibited or enhanced lysis with the exception of the B2702.60-84 peptide. This peptide blocked lysis by all CTL, regardless of their HLA specificity. This effect was due to interaction with the CTL and not the target cell as shown by pre-treatment experiments.

These peptides were tested for effects on the differentiation of CTL from CTL precursors in limiting dilution assay. The procedure was modified from Skinner and Marbrook (*J. Exp. Med.* 143:1562; 1976) as follows: PBL from normal HLA-typed donors were purified over Ficoll-Hypaque and co-cultured in round bottom microtiter wells with irradiated (10,000 R) EBV transformed B-lymphoblasts expressing the HLA allele of interest. Responder PBL were added at 3000, 6000, 10000 and 30000 cells per well while stimulators were added at 6000 cells per well. 20-4 replicates were set up for each concentration of responder cells in RPMI-1640 medium supplemented with 10% fetal bovine serum plus L-glutamine. Plates were incubated for six days in a 5% CO₂/95% air humidified incubator at which time the contents of each well were mixed by pipetting five times with a multi-channel pipette. Fifty microliter aliquots were transferred to the V-bottom microtiter wells to which 1000 ⁵¹Cr labeled targets of known HLA type were then added. Lysis was determined in a four-hour cytotoxicity assay. Wells were designated positive if specific lysis was >10%. CTL precursor frequency was determined by linear regression analysis using a computer program.

The B2702.60-84, Bw46.60-84 and Bw62.60-84 peptides all blocked the differentiation of CTL, whereas the other peptides had no effect.

25 Effect of Peptides Corresponding to HLA Regions on CTL Precursor Frequency as Determined by Limiting Dilution Analysis

Peptide	1/CTL Precursor Frequency
B2705.60-84	164,245
B2702.60-84	349,990
B38.60-84	334,937
A2.160-84	164,245
Bio46.60-84	995,400
Bio62.60-84	995,400
B27.145-169	164,245

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PBL from a normal donor (HLA-A3; B-7, 38; Cw4; DR4,6) were cultured with ty (HLA-A2; B7; DR4,6) or HOM2 (HLA-A3; B27) in the presence of 10-100 µg/ml peptide. After 6 days, lysis was tested on ⁵¹Cr-labeled CIR cells expressing either HLA-A2.5 or HLA-B2705. Results are shown for HLA-A2 specific lysis but similar results were obtained for HLA-B27 specific lysis.

The effect was not allele specific since the differentiation of CTL specific for a number of different HLA molecules was inhibited. None of the peptides affected Class II restricted responses, including mixed lymphocyte responses and mitogen induced proliferation.

PBL from normal donors were cultured at 5x10⁵ cells/round bottom microtiter well in RPMI-1640 supplemented with 10% fetal bovine serum and L-glutamine. Cultures were supplemented with either 5x10³ irradiated (10,000 R) PBV transformed B lymphoblasts or 10 µg/ml phytohemagglutinin P (PHA-P). Cells were incubated at 37°C for 3 days for PHA-P and 5 days for alloantigen at which point ³H-thymidine was added (2 µli/well). After 16 hours wells were harvested and ³H-thymidine incorporation determined by scintillation counter.

Effect of Truncated Sequences on Lysis and Differentiation

Since the B2702.60-84 and B2705.60-84 peptides differed by only 3 amino acids, additional peptides were prepared to investigate the effect of these differences. Three additional peptides were synthesized:

HLA-B2702.75-84	RENLRIALRY
HLA-B2705.75-84	REDLRTLLRY
HLA-B2702/05.60-69	WDRETQICKA

The peptide corresponding to residue 60-69 of HLA-B2702/05 had no effect on the assays described above. The peptide corresponding to residue 75-84 of HLA-B2702 blocked all Class I specific CTL responses, whereas the peptide corresponding to the same region of HLA-B2705 did not.

To determine which residue(s) mediated the inhibitory effects, 3 more peptides were synthesized in which single amino acid changes were introduced at residues 77, 80 and 81 to convert the B2702 sequence into the B2705 sequence at that position. The B2702.75-84(D) and B2702.75-84(L) peptides still blocked lysis by existing CTL and differentiation of pre-CTL while the B2702.75-84(T) peptide had no inhibitory activity. Thus, the isoleucine at position 80 is required for inhibition.

HLA-B2702.75-84(D)	REDLRIALRY
HLA-B2702.75-84(T)	RENLRITALRY

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HLA-B2702.75-84(L)

RENLRILLEY

It was also found by the following assay that B2702.60-84, B38.60-84 and B2702.75-84 when pre-bound to plastic caused cells to bind. None of the other peptides were found to have this effect. However, when the B2702.60-84 peptide was conjugated to bovine serum albumin or to beads via the cysteine at residue 67, the blocking effect and the ability to bind cells to plastics were lost.

The plastic binding procedure was as follows: peptide (100 µg/ml) was dissolved in PBS and 50 µl was added to round bottom microtiter wells or 5-10 µl to petri dishes. After 60 minutes at 37°C or overnight at 4°, the solution was removed and the plates washed twice in RPMI-1640 supplemented with 10% fetal bovine serum. Cells were added and incubated at 4° for 30 minutes. Binding to petri dishes was determined by inspecting the dishes under a microscope following gentle agitation. Binding to microtiter wells was determined after centrifugation at 500 rpm for 3 minutes. Cells which did not bind formed a small pellet at the bottom of the well whereas cells that did bind did not form a pellet.

Binding occurred equally well at 4°, 25°, or 37° and was not dependent on exogenously added divalent cations since binding was observed in medium containing EDTA. However, if cells were preincubated with 1% NaN₃ or fixed with paraformaldehyde, no binding was observed, indicating that viable cells and most likely generation of ATP were required.

Isolation and Characterization of p74

The amino terminal amino groups of the B2702.60-84, B2702.84-75-84, B2702.84-79/79-84, B2702.84-75T/75-84T, B7.60-84, and B7.84-75/75-84 peptides were conjugated to biotin-(CH)₁₂-for use with strepavidin-agarose (SAA) to isolate the peptide receptor from ³⁵S-methionine and cysteine labeled cells.

HLA-B2702.60-84	WDRETQICKAKAQTDRENLRILRY
B2702 84-75-84 Palindrome	YRLAIRLNERRENLRILRY
B2702 84-79-84 Palindrome	YRLAIRRIALRY
B2702 84-75T/75-84T Palindrome	YRLAIRLNETRENLRILRT
B7.60-84	WDRETQICKAKAQTDRESLRNLRGY
B7.84-75/75-84 Palindrome	YGRNLRLSERRESLRNLRGY

Two different protocols were used. In the first, the biotinylated peptide was complexed to the SAA and allowed to bind to labeled cells at 4°C for 30 minutes. The cells were washed free of excess complex and lysed by addition of CHAPS containing lysis buffer. This method preferentially precipitates material from the cell surface. In

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the second protocol, cell lysates were prepared in CHAPS lysis buffer and the lysate was incubated with biotinylated peptide/SAA complexes for 30 minutes at 4°C followed by extensive washing. This method preferentially precipitates intracellular material.

For both methods, proteins were separated by SDS-PAGE and visualized by fluorography. The B2702.84-75/75-84 peptide brings down 2 bands of 70 and 74 kD while the closely related B7.84-75/75-84 peptide does not. The 70 and 74 kD bands were also brought down with the B2702.60-84 and B2702.84-79/79-84 peptides but not with the B7.60-84 peptide. These two bands could be precipitated from the surface or lysate of CTL lines and peripheral blood lymphocytes but only from the lysate of most other cells tested. Substitution of either isoleucine with threonine led to a significant decrease in the intensity of both bands, and the double threonine substituted peptide, B2702.84-75T/75-84T, did not precipitate either band.

The 70 and 74 kD bands were subsequently shown to be reactive with antibodies specific for members of the heat shock protein family. Biotin-conjugated B2702.84-75/75-84 or B7.84-75/75-84 peptides were used to precipitate proteins from CTL. Following separation by SDS-PAGE, proteins were electrophoretically transferred to a nylon membrane and the Western blot was probed with a monoclonal antibody specific for Hsp70. It is seen that the antibody reacts specifically with the p74 protein.

Isoelectric focusing on the p74 protein as well as on the Hsc70 after precipitation with either peptide or anti-Hsc70 mAb shows an identical molecular weight and isoelectric points. The B2702 peptide also binds to the heat inducible form, commonly referred to as Hsp70, although the binding to the Hsc70 is much more pronounced. Both forms are expressed on the cell surface and are upregulated following treatment of cells at 43° for one hour. Expression of the Hsc70 protein correlates with peptide induced calcium increase and the induction of unresponsiveness.

Binding of the B2702 peptide to nontransformed T cells was found to result in a large calcium influx, having a magnitude and kinetics comparable to that induced by anti-CD3 antibodies, in support of the peptide binding to a surface membrane protein and being involved in the development of anergy. Other Class I HLA α 1 helix derived peptides did not induce this response under comparable conditions.

The correlation between the peptide induced increase in calcium and precipitation of the 70 and 74 kD bands from the surface for a number of different cell types is shown in Table I. The largest increase in intracellular calcium is observed in the human CTL, PBL, Jurkat, and rat spleen cells. With the exception of Jurkat, these

cells also express the highest levels of the 70 and 74 kD bands. Six μ g of p70 and p74 have been purified.

Table I. Effect of B2702.84-75-84 peptide on intracellular calcium and correlation with precipitation of 70 and 74 kD bands from the cell surface.

CELL	CALCIUM[nM]	70 kD BAND	74 kD BAND
CTL - CD8+ T cells	500	+++	+++
PBL - Human	250	++	+++
Jurkat - $\alpha\beta$ T cell tumor	400	-	+
HUT-78 - $\alpha\beta$ T cell tumor	50	-	+/-
Peer - $\gamma\delta$ T cell tumor	None	-	-
HSB - $\alpha\beta$ T cell tumor	None	-	-
NK clones - Human	200	ND	ND
JY - B lymphoblast	None	-	+/-
MS - Burkitt's	100	-	+
Daudi - β_2m -Burkitt's	75	-	+
K562 - Pre-erythrocyte	None	-	-
HEL - Pre-erythrocyte	None	-	-
Rat lymphocytes -ACI spleen	200	-	+++

- An increase in intracellular Ca^{2+} induced by peptide was observed in only some of the cell lines tested. The greatest changes in intracellular Ca^{2+} were demonstrated in PBL, CTL lines, and the human T cell tumor Jurkat. A smaller but significant increase in intracellular Ca^{2+} was induced by the inverted repeat B2702.84-75/75-84 peptide in two Burkitt's lymphoma cell lines, Daudi and MS. The B2702.84-75/75-84 peptide did not cause a Ca^{2+} flux in the human T cell lines Peer, HUT-78, HSB; human Epstein Barr virus transformed B cell lines, including JY and 721.221; the natural killer cell line YT2C2; or several pre-erythrocytic cell lines, including K562 and HEL.
- Neither the B2702.75-84 peptide nor the inverted repeat B7.84-75/75-84 peptide initiated Ca^{2+} mobilization in any of the cells tested.

- The threonine substituted peptides, which failed to cause unresponsiveness in T cells, were also unable to promote Ca^{2+} mobilization. The maximum intracellular Ca^{2+} level induced by the B2702.84-75/75-84T and B2702.84-75T/75-84 peptides was only 10-30% of that achieved with the unsubstituted B2702.84-75/75-84 peptide. The double substituted B2702.84-75T/75-84T peptide did not initiate any Ca^{2+} flux. These results suggest that the B2702 peptides cause an increase in intracellular Ca^{2+}

-15-

that results in T cell unresponsiveness. The B2702.84-75T/75-84T peptide, which did inhibit any T cell responses, did not cause a rise in intracellular Ca^{2+} .

Mobilization of intracellular Ca^{2+} is mediated by at least two major mechanisms. IP_3 is one messenger molecule that links surface receptor activation to the release of Ca^{2+} from internal stores by binding to a receptor on the endoplasmic reticulum. The level of IP_3 in cells is elevated following the activation of either G protein linked receptors or by tyrosine kinase linked receptors. The other major mechanism is Ca^{2+} induced Ca^{2+} release (CICR). It has recently been demonstrated that a molecule called cyclic adenosine diphosphate ribose (cADPR), a metabolite of nicotinamide adenine dinucleotide (NAD^+), is the messenger responsible for regulating this channel through the ryanodine receptor. Using the T cell line Jurkat, we observed that stimulation with anti-TCR mAb produced a substantial increase in IP_3 whereas treatment with the B2702.84-75/75-84 peptide had no effect on levels of IP_3 . The peptide induced increase in intracellular Ca^{2+} is likely to be mediated through the ryanodine receptor.

Another early event in T cell activation is a change in the patterns of phosphorylation of many proteins. Therefore, it was determined whether the peptides that inhibited T cell function affected protein phosphorylation or the level of intracellular calcium. None of the peptides examined thus far has had any effect on the pattern of tyrosine phosphorylation observed by Western blot analysis in whole lysates from T cells.

A comparison of the effects of the B2702 84-75-84 palindrome with other known immunosuppressants demonstrates a novel pattern of activity. The results are summarized in Table 2.

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Table 2

	CSA	FK506	Rapamycin	deoxyspergualin	B2702
Block T cell proliferation	+++	+++	+++	+++	+
Block Cytotoxicity	-	-	-	-	+++
Block Cytokine Transcription	+++	+++	-	ND	ND
Block IL-2 Transcription	+++	ND	-	ND	ND
Induce Ca++ flux	-	-	-	-	+++
Block calcineurin	+++	+++	-	ND	ND
Cellular Receptor	Cyclophilin	FKBP	FKBP	Hsc70	p74

5 The data demonstrates that B2702 has a novel pattern of activity, as compared to the known immunosuppressants, cyclosporin A, FK506, rapamycin and deoxyspergualin.

It is evident from the above results, that p74 plays an important role in the modulation of CTL activity. By controlling protein interactions resulting in signal transduction or the absence of signal transduction, CTL differentiation and cytolytic activity can be modulated.

10 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

15 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: The Board of Trustees for the Leland Stanford Junior University
- (ii) TITLE OF INVENTION: SURFACE MEMBRANE PROTEINS AND THEIR EFFECT ON IMMUNE RESPONSE
- (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: FLEHR, HOHBACH, TEST, ALBRITTON & HERBERT
 - (B) STREET: 4 Embarcadero Center, Suite 3400
 - (C) CITY: San Francisco
 - (D) STATE: California
 - (E) COUNTRY: USA
 - (F) ZIP: 94111-4187
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: PCT/US94/
 - (B) FILING DATE: 10-NOV-1994
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/150,493
 - (B) FILING DATE: 10-NOV-1993
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Rowland, Bertram I.
 - (B) REGISTRATION NUMBER: 20,015
 - (C) REFERENCE/DOCKET NUMBER: FP-58976/BIR
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (415) 781-1989
 - (B) TELEFAX: (415) 398-3249
 - (C) TELEX: 910 277299

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Arg Leu Ala Ile Arg Leu Asn Glu Arg Glu Asn Leu Arg Ile			
1	5	10	15
Ala Leu Arg Tyr			
20			

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids

-18-

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Tyr Gly Arg Leu Asn Arg Leu Ser Glu Arg Arg Glu Ser Leu Arg Asn
1           5           10           15

Leu Arg Gly Tyr
                20

```

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

Trp Asp Arg Glu Thr Gln Ile Cys Lys Ala Lys Ala Gln Thr Asp Arg
1           5           10           15

Glu Asn Leu Arg Ile Ala Leu Arg Tyr
                20           25

```

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Tyr Arg Leu Ala Ile Arg Leu Asn Glu Arg Arg Glu Asn Leu Arg Ile
1           5           10           15

Ala Leu Arg Tyr
                20

```

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Tyr Arg Leu Ala Ile Arg Arg Ile Ala Leu Arg Tyr
1           5           10

```

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Leu Arg Thr
20

(2) INFORMATION FOR SEO ID NO:7:

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Ser Leu Arg Asn Leu Arg Gly Tyr
20 25

(2) INFORMATION FOR SEQ ID NO:8:

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Arg Gly Tyr
20

(2) INFORMATION FOR SEQ ID NO:9:

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

-20-

Arg Glu Asp Leu Arg Ile Ala Leu Arg Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Arg Glu Asn Leu Arg Thr Ala Leu Arg Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Arg Glu Asn Leu Arg Ile Leu Leu Glu Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Arg Glu Asn Leu Arg Ile Ala Leu Arg Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Arg Glu Asp Leu Arg Thr Leu Leu Arg Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids

-21-

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Trp	Asp	Arg	Glu	Thr	Gln	Ile	Cys	Lys	Ala
1				5					10

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Trp	Asp	Arg	Glu	Thr	Gln	Ile	Cys	Lys	Ala	Lys	Ala	Gln	Thr	Asp	Arg
1				5				10						15	
Glu	Asp	Leu	Arg	Thr	Leu	Leu	Arg	Tyr							
			20					25							

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Trp	Asp	Arg	Glu	Thr	Gln	Lys	Tyr	Lys	Arg	Gln	Ala	Gln	Thr	Asp	Arg
1				5				10						15	
Val	Ser	Leu	Arg	Asn	Leu	Arg	Gly	Tyr							
			20					25							

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Trp	Asp	Arg	Glu	Thr	Gln	Ile	Ser	Lys	Thr	Asn	Thr	Gln	Thr	Tyr	Arg
1				5				10						15	
Glu	Ser	Leu	Arg	Asn	Leu	Arg	Gly	Tyr							
			20					25							

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:

-22-

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```

Trp Asp Gly Glu Thr Arg Lys Val Lys Ala His Ser Gln Thr His Arg
1          5          10          15
Val Asp Leu Gly Thr Leu Arg Gly Tyr
          20          25

```

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```

Arg Lys Trp Glu Ala Ala Arg Val Ala Glu Gln Leu Arg Ala Tyr Leu
1          5          10          15
Glu Gly Glu Cys Val Glu Trp Leu Arg
          20          25

```

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```

Trp Asp Arg Asn Thr Gln Ile Cys Lys Thr Asn Thr Gln Thr Tyr Arg
1          5          10          15
Glu Asn Leu Arg Ile Ala Leu Arg Tyr
          20          25

```

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WHAT IS CLAIMED IS:

1. A composition comprising at least 50 weight % of a lymphoid surface membrane protein designated as p74 and characterized by:
 - 5 having an affinity to HLA-B2702.84-75-84 peptide of at least about 10^{-4} M, having a molecular weight of about 74 kD as determined by SDS-PAGE gel electrophoresis,
 - being expressed on B and T cells; and
 - when present as a surface membrane protein of a nontransformed T cell upon
 - 10 binding to HLA-B2702.60-84 induces calcium influx and inhibits at least one of CTL differentiation or cytolysis by said T cell, or fragment thereof comprising at least the portion of said protein binding to said HLA-B2702.84-75-84, the transmembrane region, the intracellular region or the extracellular region.
- 15 2. A composition according to Claim 1 comprising a fragment of said protein according to Claim 1, comprising said extracellular region and lacking a transmembrane region.
- 20 3. A composition according to Claim 1, comprising at least 90 weight % of said protein.
4. A composition according to Claim 1, wherein said protein is obtained by affinity purification with a polypeptide comprising a Bw4 epitope.
- 25 5. A method of screening compounds for their effect on cytolytic activity of T cells, said method comprising:
 - combining a candidate compound with the extracellular portion of p74; and
 - determining the amount of binding of said compound to p74 .
- 30 6. A method according to Claim 5, wherein said extracellular portion of p74 is present on the surface membrane of a T cell.
7. A method according to Claim 4, wherein said binding is related to the cytolytic activity of said T cell.
- 35

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8. A method for enriching a composition for p74 from a lysate from cells expressing p74, said method comprising:

combining said lysate with the palindromic peptide HLA-B2702.84-75-84 bound to a solid support;

5 separating said lysate from said solid support;

washing said solid support to remove non-specifically bound proteins; and

isolating protein bound to said support to provide a composition enriched for p74.

10 9. A method for inhibiting the modulation of CTL activity of in a cellular composition comprising T cells and antigen presenting cells (APC), said method comprising:

adding to a cellular mixture of CTLs and APCs the extracellular portion of p74 free of cellular surface membrane in an amount sufficient to compete with p74 for

15 binding of ligand to p74,

whereby the activity of said CTLs is modulated.

10. A method according to Claim 9, wherein an immunosuppressant is added to said cellular mixture up to an amount to immunosuppress said CTLs.

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US94/12985

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 1/22, 14/705, 14/725; G01N 33/566

US CL : 435/7.24, 7.8; 436/501; 530/350, 413

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.24, 7.8; 436/501; 530/350, 413

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

The Leucocyte Antigen Facts Book, Barclay et al, Academic Press Limited, 1993

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Proceedings of the National Academy of Sciences USA, Vol.91, issued March 1994, CIAVARRA ET AL., "Heat stress induces hsc70/nuclear topoisomerase I complex formation <i>in vivo</i> : Evidence for hsc70-mediated ATP-independent reactivation <i>in vitro</i> ", pages 1751-1755. See page 1752, col. 1, lines 2-3.	1-4
A	Biological Abstracts, Vol. 87, No. 2, issued January 1991, HAIRE ET AL., "Mitogen-induced preferential synthesis of proteins during the G0 to S phase transition in human lymphocytes". See page 182, col. 2, the abstract no. 14222. Experimental Cell Research, 1988, 179(1) 65-78. See entire abstract.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:	
A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
B earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	*A* document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 FEBRUARY 1995

Date of mailing of the international search report

17 FEB 1995

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

Box PCT

Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DAVID SAUNDERS

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/12985

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-8

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS searches:

- 1) HSC70 or HSC(W)70 or heat(W)shock(W)(cognate or constitutive)(SW)70 and HLA(W)B27?
- 2) " " " " " " " " and (B or T)(W)
(cell# or lymphocyt?)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-8, drawn to a p74 protein, a methods of its preparation, and a method of its use in screening assays.
- II. Claims 9-10, drawn to a method of use of the p74 protein in modulating T-cell activity.

The first recited product, process of its manufacture, and method of use are considered as the main invention. See PCT Article 17(3)a). Instantly, the additional use recited in Group II does not fall within the main invention. The use recited in Group II involves no step in common with that of the first recited use in claims 5-7 - i.e. in the use of Group II p74 is added as an extracellular composition (claim 9, lines 4-5), while in the use of Group I p74 is on the cell membrane (claim 6, line 2). Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.